Thermal Resistance of *Aeromonas hydrophila*

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ABSTRACT

Thermal resistance of five strains of *Aeromonas hydrophila* (three clinical and two food isolates) was studied at 45 to 51°C in saline solution and raw milk. In addition, effects of growth temperature and growth phase on thermal resistance of the cells were also studied. Survivors after various heat treatments were plated on starch phenol red agar; colonies were counted after 24 h at 28°C. Cells heated at 48°C and 51°C exhibited a diphasic response and the data presented are from the initial and final linear phases. Data were expressed as D- and z-values. Most variables caused small but statistically significant changes in D-value of the initial linear phase. At 48°C, D-values for stationary phase cells heated in saline solution ranged from 3.49 to 6.64 min; for cells heated in raw milk, the D-values ranged from 3.20 to 6.23 min. At 48°C, D-values for log-phase cells heated in saline solution ranged from 2.23 to 3.73 min, and z-values ranged from 5.22 to 7.69°C. These results indicate that *A. hydrophila* should be killed by many of the heat treatments given foods during processing. The thermal resistance of *A. hydrophila* appears similar to that of other gram-negative bacteria associated with food.

Aeromonas hydrophila is becoming recognized as a human pathogen and is being isolated with increasing frequency from cases of human diarrhea *(6,14,15,25)*. A recently completed survey *(19)* of retail fresh foods of animal origin (fish and seafood, red meat, poultry, raw milk) determined that the organism was present in virtually every food sampled, often at levels of greater than 10^5/g or ml. Though the organism has been described in the older literature *(8)*, especially as a pathogen of fish and reptiles, nothing is known about its behavior under food processing conditions, particularly its response to heat. We thus became interested in determining the heat resistance of this emerging psychrotrophic pathogen and the influence of factors such as phase of growth, heating menstruum, and temperature of growth of the cells on this heat resistance. This last factor is particularly important since a study from this laboratory *(20)* has indicated that clinical isolates can grow over a temperature range of 4°C to 42°C and that the organism can grow competitively in foods held at 5°C *(19)*.

MATERIALS AND METHODS

Organisms

Five strains of *A. hydrophila* were used in these studies: three clinical strains (K144, BA2, BW37) *(20)* and two strains (B2-10 and F6-10) isolated from foods in our laboratory *(19)*.

Growth conditions

The organisms were grown in tryptic soy broth (Difco, 100 ml/500-ml flask) and incubated with aeration (200 rpm). The cultures were grown at 37, 28, 19 and 5°C to stationary phase *(20)*; in addition, cells were grown to log phase (ca 3-4 h) at 28°C. After growth, cells were harvested by centrifugation (13,000 × *g* 10 min, 5°C), and then resuspended in 3 ml of sterile saline solution just before addition to the heating menstruum.

Heating conditions

The effect of most variables was studied by heating at 48°C. To determine z-values, cultures were also heated at 45 and 51°C. For most studies, cells were heated in saline (0.85%) solution; raw milk was used in one study. The heating menstruum was placed in a sterile 1-qt. mason jar, closed with a rubber stopper and held in a constant temperature water bath, stirred continuously, and allowed to equilibrate to the selected temperature. The heating menstruum occupied about 1½" in the heating container; the level of fluid in the jar was about 2" below the surface of the water in the bath. Foaming was minimal. After the desired temperature was achieved, the resuspended cells were added. The time to return to the test temperature was minimal. The temperature of the heating menstruum was monitored continuously by a thermocouple probe inserted into the liquid. The probe was connected to a YSI model 42SC Tele-Thermometer. Periodic calibration checks of the system were made by attaching the probe to a mercury thermometer and adjusting the setting of the YSI if needed.

Survivors

At intervals, portions were removed, diluted in 0.1% peptone water as needed, and surface-plated on starch phenol red agar using a Spiral plater (model DU, Spiral Systems, Bethesda, MD). Colonies were counted after 24 h at 28°C.

Data

Survivor plots (log viable *A. hydrophila*/ml vs. time) were determined for all variables and best fit regression lines calculated. D-values were calculated as the negative reciprocal of the slope obtained from regression analysis. z-Values were ob-

tained from a plot of log D-value vs temperature and expressed as °C to bring about a 10-fold change in D-values. All experiments were done twice in duplicate, thus each D-value in Tables 1-3 is the result of 4 replicates. Statistical analyses, including ANOVA, of the effect of variables on D-values, were performed using SAS procedures *(23)*.

RESULTS

Plating medium

In preliminary experiments, several plating media were evaluated. These included nutrient agar (NA, Difco), tryptic soy agar (TSA, Difco), TSA + Congo Red (10 mg/L, TSACr), and phenol red starch ampicillin agar (SAA, *19)*. Plates were counted at both 24 and 48 h. The additional 24 h did not increase the number of survivors, so 24 h incubation was used in all further experiments. Comparison of counts obtained from the four media indicated that with unheated cells, the counts were the same. With cells heated at 45°C, especially for periods longer than 45 min, SAA gave better recovery than NA and as good as TSA and TSACr. Since we had previous experience with SAA in the quantitative recovery of *A. hydrophila* from foods *(19)*, we chose SAA for use in this study. In addition, we omitted the ampicillin because it served no useful purpose since we were studying pure cultures. In the series of experiments in which raw milk was the heating menstruum, the presence of *A. hydrophila* after heating was verified by flooding the plates with Lugol's iodine and counting only amylase-positive colonies *(19)*.

Temperature of heating

The survivor plots for *A. hydrophila* BA2 heated at 45, 48, and 51°C in saline solution are given in Fig. 1. Analysis of the respective correlation coefficients indicated that a linear response adequately/properly described the fit for cells heated at 45°C. Visually, the plots for 48 and 51°C appear to have two distinct linear phases and the correlation coefficients (r) for 45°C heating temperature averaged 0.96 for the five strains studied and

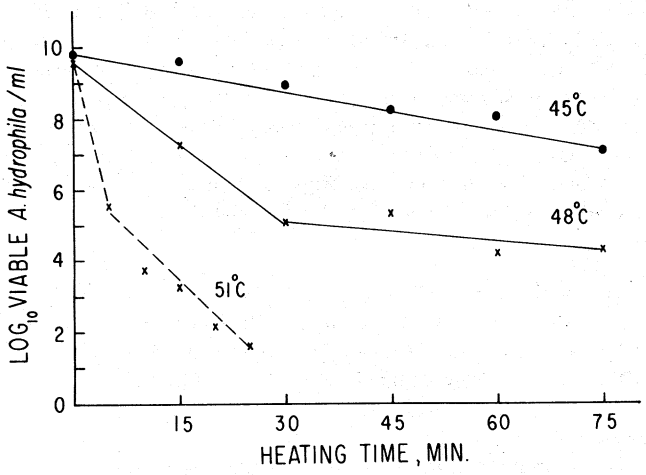

Figure 1. *Effect of heating temperature on D-values for* A. hydrophila *BA-2. 45°C: D=27.45 min, 48°C: D=6.30 min (initial phase), 51°C: D=1.14 min (initial phase).*

decreased to 0.76 for 51°C. An r of 0.76 is below 0.81 (95% confidence level), indicating a non-linear fit of the data (Calculator Decision-Making Sourcebook, Texas Instruments, 2nd ed, 1981.). The 48 and 51°C results were then analyzed by a multiple regression analysis technique *(7)*, which indicated that the fit of the data can be described by two straight lines. The D-values for the three heating temperatures are presented in Table 1a and Table 1b along with the z-values for the initial phase.

The effect of other variables on heat resistance of *A. hydrophila* strains was studied at 48°C. These D-values are presented in Tables 1a and 1b, 2, and 3. Comparison of the effect of the variables in Tables 1, 2, and 3 indicated that most variables had only small effects on heat resistance.

The D-value data in Tables 1, 2 and 3 (for the initial linear phase) were subjected to analysis of variance (ANOVA) to determine the possible effect(s) of growth temperature, strain differences, strain origin, growth phase, and heating menstruum on heat resistance of *A. hydrophila*. Statistically significant differences ($P=0.05$) were obtained, but no consistent distinct pattern was observed. The results of these analyses are as follows: *(a)* using orthogonal contrasts in the ANOVA, raw milk was compared with saline solution as heating menstruum--D-values for saline solution were significantly higher than those for raw milk for strains BW37, K144, and F6-10, but there was no difference for BA2 and B2-10; *(b)* again using orthogonal contrasts in the ANOVA, D-values for log phase cells were significantly lower than those for stationary phase cells except for B2-10 and F6-10, where there was no difference; *(c)* for the effect of growth temperature on heat resistance, data were analyzed by analysis of covariance. This analysis yielded evidence of a linear increase of D-value with increasing growth temperature for strains BA2 and BW37, but there was no evidence for such an effect for other strains; *(d)* for comparing clinical and food isolates, the data were also analyzed by analysis of covariance; this indicated that the decrease of D-value with increased heating temperature was significantly less pronounced in the food isolates than in the clinical isolates except for strain K144; and *(e)* for comparing the effect of heating temperature on the response of clinical and food isolates, the data were analyzed by analysis of covariance; this analysis indicated that the z-values for food isolates were significantly lower than those for the clinical isolates.

DISCUSSION

The heat resistance (D-values for the initial linear phase - Tables 1, 2, and 3) of *A. hydrophila* strains studied are in line with D-values for other gram-negative bacteria associated with food. Kaufmann and Andrews *(11)* reported $D_{50°C}$ of 1.1 min for *Pseudomonas viscosa* and $D_{49.4°C}$ of 1.4 min for culture 29 (a gram-negative psychrotroph isolated from refrigerated milk). Koidis and Doyle *(12)* reported a $D_{50°C}$ of 6.1 min for *Campylobac-*

TABLE 1. *Effect of heating temperature and growth temperature on the D- and z-values for five strains of* A. hydrophila *(heated in saline solution).*

a. Initial Phase

		Strains				
Growth temp. (°C)	Heating temp. (°C)	K144	BA2	BW37	B2-10	F6-10
28	45	13.84 ± 1.78[a]	29.54 ± 2.57	25.97 ± 2.14	12.01 ± 2.64	13.47 ± 1.16
28	48	5.54 ± 0.66	5.50 ± 0.47	6.64 ± 1.22	3.49 ± 0.35	4.64 ± 1.56
28	51	1.23 ± 0.05	2.34 ± 0.30	1.80 ± 0.30	1.76 ± 0.41	2.22 ± 0.49
z-value	°C	5.71	5.45	5.22	6.98	7.69
37	48	3.64 ± 0.50	7.76 ± 1.03	9.93 ± 0.72	3.73 ± 0.33	4.35 ± 0.80
19	48	3.28 ± 0.15	3.33 ± 0.57	4.12 ± 0.71	3.83 ± 0.40	4.29 ± 1.34
5	48	3.39 ± 0.72	3.50 ± 0.60	3.29 ± 0.67	2.84 ± 0.27	2.75 ± 0.54

b. Final Phase

		Strains				
Growth temp. (°C)	Heating temp. (°C)	K144	BA2	BW37	B2-10	F6-10
28	48	42.57 ± 13.68[a]	63.39 ± 57.04	156.55 ± 148.49	31.83 ± 12.39	36.43 ± 14.74
28	51	30.04 ± 12.63	8.08 ± 3.72	23.25 ± 7.95	46.58 ± 44.74	122.68 ± 120.55
37	48	26.90 ± 9.94	63.98 ± 53.93	15.35 ± 0.95	38.87 ± 16.41	33.24 ± 2.49
19	48	78.55 ± 63.28	31.30 ± 8.46	43.13 ± 25.35	43.53 ± 27.01	75.68 ± 89.00
5	48	42.99 ± 5.13	31.48 ± 6.41	44.13 ± 31.91	28.39 ± 4.35	132.13 ± 113.25

[a]D-value (in minutes) followed by SD (n = 4).

TABLE 2. *D. values for five strains of* A. hydrophila *grown to the exponential phase (grown at 28°C and heated at 48°C in saline solution).*

	Strains				
Phase	K144	BA2	BW37	B2-10	F6-10
Initial	2.23 ± 0.41[a]	2.57 ± 0.11	3.49 ± 0.57	3.65 ± 0.15	3.73 ± 0.91
Final	96.93 ± 98.61[a]	31.20 ± 15.89	19.55 ± 2.93	390.51 ± 531.63	93.36 ± 126.98

[a]D-value (in minutes) followed by SD (n = 4).

TABLE 3. *D-values for five strains of* A. hydrophila *heated in raw milk (grown at 28°C and heated at 48°C).*

	Strains				
Phase	K144	BA2	BW37	B2-10	F6-10
Initial	4.10 ± 0.74[a]	6.23 ± 1.31	4.73 ± 0.22	3.20 ± 0.66	3.31 ± 0.72
Final	67.90 ± 20.04[a]	28.39 ± 4.35	36.45 ± 18.86	131.11 ± 118.82	326.29 ± 529.33

[a]D-value (in minutes) followed by SD (n = 4).

ter jejuni in ground beef, while Blankenship and Craven *(2)* found a $D_{51°C}$ of 8.77 min for the same organism in ground chicken meat. *Escherichia coli, Yersinia enterocolitica,* and *Salmonella manhattan* are somewhat more heat resistant with the following values: $D_{51.7°C}$ of 28.2 to 39.3 min for various dairy products, $D_{50°C}$ of 2.56 to 57.3 min for skim milk, and $D_{54.4°C}$ of 7.1 and 14.4 min for chicken a la king and custard respectively *(1,9,22)*. The corresponding heat resistance for *A. hydrophila* strains are $D_{48°C}$ of 3.2 to 6.2 min for raw milk (Table 3).

Hansen and Riemann *(10)* reported that the z-values [the relationship between log D-value and temperature (°C)] for most nonsporeforming bacteria lie between 4 and 6°C. The z-values for *A. hydrophila* (Table 1a) are both within this range and above it. However, for the same gram-negative bacteria mentioned above, there is also a range of z-values. Angelotti et al. *(1)* calculated a z-value of 7.64 and 5.2°C for *S. manhattan* in custard and chicken a la king and Read et al. *(22)* found a z-value of 5.65°C for *E. coli* heated in various dairy products. Kaufmann and Andrews *(11)* calculated a z-value of 7.8°C for *P. viscosa* and 3.7°C for culture 29, both in skim milk. Using the D-value data of Blankenship and Craven *(2)* for *C. jejuni* heated in ground chicken meat, a z-value of 5.8°C was calculated.

Tomlins and Ordal *(24)* have surveyed the literature on the effect of heat on vegetative mesophilic bacteria and found z-value ranges similar to those of Hansen and Riemann *(10)*. However, values above 6.0 were encountered, though these were usually found in conjunction with complex heating systems such tomato juice, broth

with lowered water activity, milk chocolate, and egg yolk plus 10% NaCl. A few organisms with z-values of less than 4 were also found *(24)*.

The major problem encountered in this study is one which has plagued microbiologists almost from the first thermal death time study: non-linear response of the organism to increasing amounts of heat. The topic of non-linear response has been thoroughly reviewed on many occasions *(3,4,5,13,16,17,18)*. Analysis of a non-linear response has always been difficult.

Heating at 45°C yielded a linear response over the time period studied (Fig. 1). This made calculation of D-values straight forward (Table 1). At 48 and 51°C, plots with two distinct linear phases are observed (Fig. 1). This diphasic response to heat is in contrast to the organism's response to radiation. A recent study *(21)* from this laboratory indicated that a linear response (log viable number vs. dose) properly described radiation killing for *A. hydrophila (21)*.

The diphasic response appears to be inherent in the organism. When a culture from cells heated for 75 min at 48°C was regrown and reheated at 48°C, it also exhibited a diphasic response. Moats *(18)* indicated that variation in heat resistance of the population remains the only acceptable explanation of concave survivor curves. The time at which the culture shifted from one linear phase to another occurred earlier at higher heating temperatures (Fig. 1.); however, the shift occurred at about the same population level. A separate experiment with strain B2-10 determined that, if heated at 45°C for an extended period (up to 120 min), a breakpoint did occur (at a population of 10^5-10^6; data not presented). Vos and Proszt *(26)* have suggested that a mixed resistance population is probably the norm. With spores of *Bacillus cereus,* they determined that one cell in 10^7 or 10^8 possessed extreme heat resistance. With *A. hydrophila,* this fraction appears to be somewhat higher.

Considerable difficulty was encountered when we attempted to calculate D-values for the second linear phase of the killing curve. In some instances, the second portion was virtually flat. In others, there were large variations among the four replicates (Tables 1a and 1b, 2, and 3). Moats et al. *(18)* discussed the problems of "tailing" of thermal death plots and presented observations similar to ours for the second linear phase. They presented data for a strain of *E. coli* which showed a rapid initial decrease in count when heated at 62.5°C, followed by no further decrease between 5 and 60 min of heating.

In analyzing our data, we chose a diphasic linear model, a model which was supported by analyzing the data by a multiple regression analysis technique *(7)*. For comparison of the variables, we compared D-values for the initial linear phase. These comparisons indicated relatively small though statistically significant differences attributed to growth temperature of the cells, heating menstruum, and growth phase of the cells (Table 1).

The data presented here support the concept that the response of *A. hydrophila* to heat can be described by a diphasic linear plot. D-values (and z-values) obtained from the initial linear phase indicated that the heat resistance of *A. hydrophila* is comparable to that of other gram-negative bacteria associated with food, and thermal processes designed to eliminate *Salmonella* should be sufficient to inactivate *A. hydrophila*. The significance of the heat resistant subpopulation is not known and is being subjected to further study.

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